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# EXPRESSED PROTEIN LIGATION. A NEW TOOL FOR THE BIOSYNTHESIS OF CYCLIC POLYPEPTIDES

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# EXPRESSED PROTEIN LIGATION. A NEW TOOL FOR THE BIOSYNTHESIS OF CYCLIC POLYPEPTIDES

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**Abstract.** The present paper reviews the use of expressed protein ligation for the biosynthesis of backbone cyclic polypeptides. This general method allows the *in vivo* and *in vitro* biosynthesis of cyclic polypeptides using recombinant DNA expression techniques. Biosynthetic access to backbone cyclic peptides opens the possibility to generate cell-based combinatorial libraries that can be screened inside living cells for their ability to attenuate or inhibit cellular processes.

**Keywords:** Circular proteins, Protein splicing, Intein-mediated ligation, Native Chemical Ligation, Protein engineering, Polypeptide libraries

## Introduction

A significant number of natural products with wide range of pharmacological activities derive from cyclic polypeptides. In fact, peptide cyclization is widely used in medicinal chemistry to improve the biochemical and biophysical properties of peptide-based drug candidates [1,2]. Cyclization rigidifies the polypeptide backbone structure, thereby minimizing the entropic cost of receptor binding and also improving the stability of the topologically constrained polypeptide. Among the different approaches used to cyclize polypeptides, backbone or head-to-tail cyclization remains one of the most extensively used to introduce structural constraints into biologically active peptides.

Despite the fact that the chemical synthesis of cyclic peptides has been well explored and a number different approaches involving solid-phase or liquid-phase exist [3-7], recent developments in the fields of molecular biology and protein engineering have now made possible the biosynthesis of cyclic peptides. This progress has been made mainly in two areas, non-ribosomal peptide synthesis [8-10] and expressed protein ligation/protein trans-splicing [11-16]. The former strategy involves the use of genetically engineered non-ribosomal peptide synthetases and is reminiscent of more established technologies that yield novel polyketides. The later strategy

relies on the heterologous expression of recombinant proteins fused to modified intein protein splicing/trans-splicing units [17].

The biosynthesis of cyclic polypeptides offers many advantages over purely synthetic methods. Using the tools of molecular biology, large combinatorial libraries of cyclic peptides, may be generated and screened *in vivo*. A typical chemical synthesis may generate $10^4$ different molecules. It is not uncommon for a recombinant library to contain as many as $10^8$ members. The molecular diversity generated by this approach is analogous to phage-display technology. Moreover, this approach takes advantage of the enhanced pharmacological properties of backbone-cyclized peptides as opposed to linear peptides or disulfide-stabilized polypeptides. Also, the approach differs from phage-display in that the backbone-cyclized polypeptides are not fused to or displayed by any viral particle or protein, but remain on the inside of the living cell where they can be further screened for biological activity. The complex cellular cytoplasm provides the appropriate environment to address the physiological relevance of potential leads.

Protein trans-splicing has been succesfully used by Benkovic and co-workers to generate backbone cyclized or polypeptides *in vivo* [12]. In this approach, the peptide to be cyclized is nested between the two split intein fragments of the naturally occurring *Ssp* DnaE split intein [18] (usually referred as *N*- and *C*-inteins) in such way that the *N*-terminus of the peptide template is fused to *C*-intein fragment and *vice versa*. Protein splicing of this chimeric protein leads to the formation of the desired cyclic peptide inside *E. coli* cells. A potential limitation of this approach, however, is the requirement for specific *N*- and *C*-extein residues at the intein junction sites [19]. These amino acids are necessary for efficient protein splicing to occur, which restricts the sequence diversity within the sequence of the cyclic peptide,

An attractive alternative approach to the biosynthesis of circular polypeptides is the use of an intramolecular version of Native Chemical Ligation reaction [20-22]. The present paper reviews the use of this reaction for the biosynthesis of circular polypeptides (i.e. peptides and proteins) and it will discuss also the potential of this method for the biosynthesis of cyclic polypeptide libraries inside living cells.

**Native Chemical Ligation**

Native Chemical Ligation (NCL) is an exquisitely specific ligation reaction that has been extensively used for the total synthesis, semi-synthesis and engineering of different proteins [21,23-25]. In this reaction, two fully unprotected polypeptides, one containing a *C*-terminal α-thioester group and the other a *N*-terminal Cys residue, react chemoselectively under neutral aqueous conditions with the formation of a native peptide bond (Figure 1A). The initial step in this ligation involves the formation of a thioester-linked intermediate, which is generated by a trans-thioesterification reaction involving the α-thioester moiety of one fragment and the *N*-terminal Cys thiol group of the other fragment. This intermediate then spontaneously rearranges to produce a peptide bond at the ligation site. This type of thioester-based chemistry was first discovered by Wieland in 1950's for the synthesis of small Cys-containing peptides [26,27].

It is well established that when these two reactive groups, i.e. the *C*-terminal α-thioester group and the *N*-terminal Cys residue, are located in the same synthetic precursor, the chemical ligation proceeds in an intramolecular fashion thus resulting in the efficient formation of a circular polypeptide (Figure 1B). These reaction has been successfully employed for the chemical synthesis of cyclic peptides and small protein domains [3,5-7].

**Expressed Protein Ligation.** Recent advances in protein engineering have made also possible the introduction of the *C*-terminal α-thioester group and *N*-terminal Cys residue into recombinant proteins. These important developments make possible the use of NCL between synthetic and/or recombinant fragments. This new technology, called Expressed Protein Ligation (EPL), now allows access to a multitude of chemically engineered recombinant proteins including biosynthetic circular polypeptides [25].

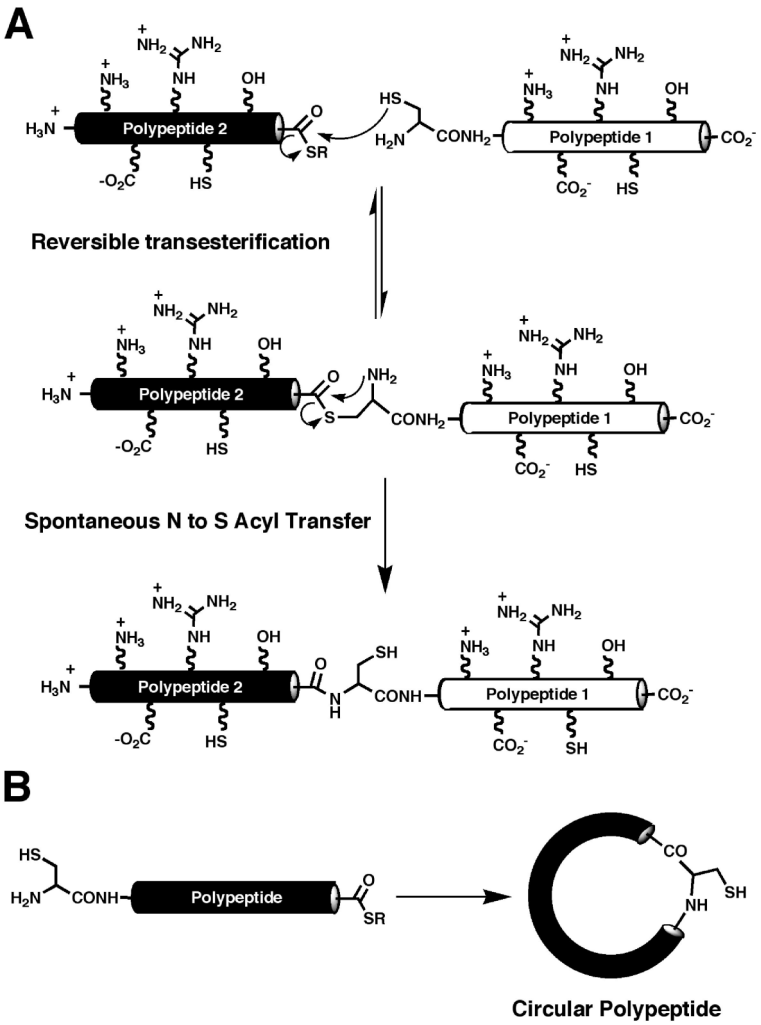


***Figure 1**. **A**. Principle of Native Chemical Ligation (NCL). **B**. Intramolecular NCL leads to the formation of backboned cyclized polypeptides*

*Recombinant polypeptide α-thioesters.* Recombinant protein α-thioesters can be obtained by using engineered inteins [24,28-30]. Inteins are self-processing domains which mediate the naturally occurring process called protein splicing [31] (Figure 2). Protein splicing is a cellular processing event that occurs post-translationally at the polypeptide level. In this multistep process an internal polypeptide fragment, called intein, is self-excised from a precursor protein and in the process ligates the flanking protein sequences (*N*- and *C*-exteins) to give a different protein. The current understanding of the mechanism is summarized in Figure 2 and involves the formation of thioester/ester intermediates [31]. The first step in the splicing process involves an N→S or N→O acyl shift in which the *N*-extein is transferred to the thiol/alcohol group of the first residue of the intein. After the initial N→(S/O) acyl shift, a trans-esterification step occurs in which the *N*-extein is transferred to the side-chain of a second conserved Cys, Ser or Thr residue, this time located at the junction between the intein and the *C*-extein. The amide bond at this junction is then broken as a result of succinimide formation involving a conserved Asn residue within the intein. In the final step of the process, a peptide bond is formed between the *N*-extein and *C*-

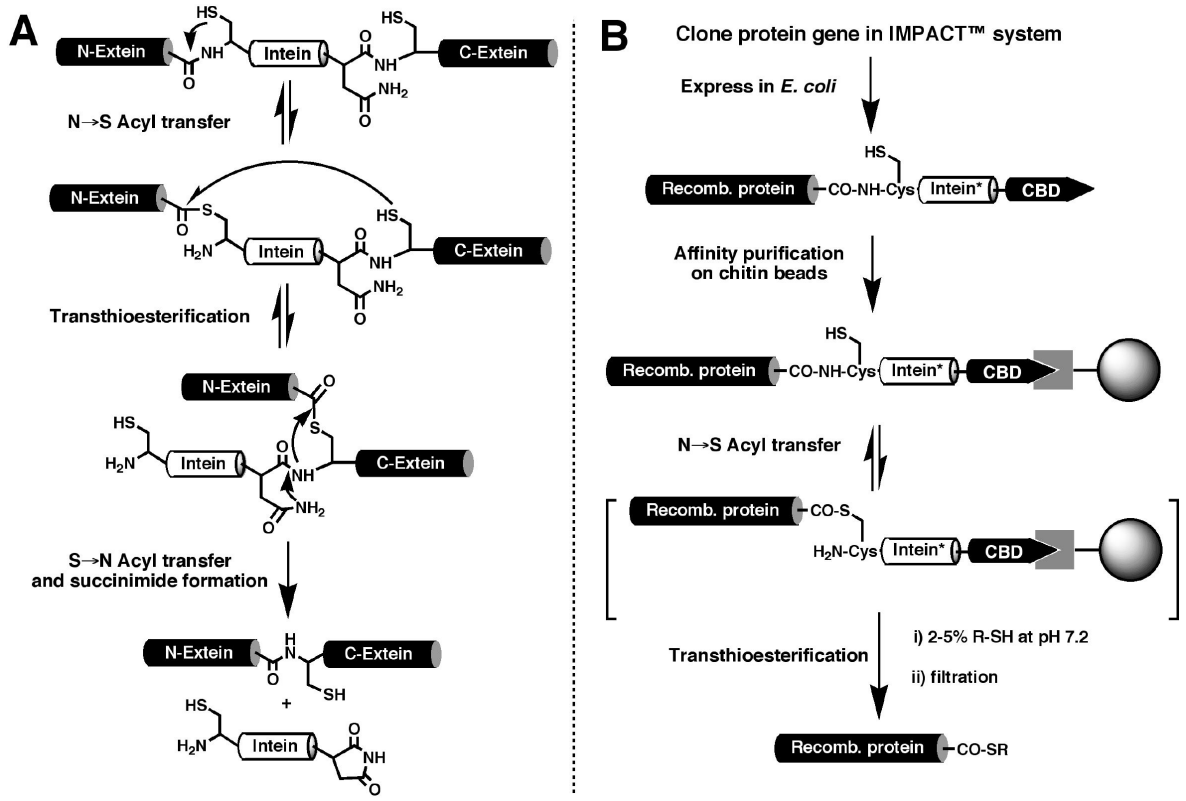


***Figure 2.*** *Biosynthesis of recombinant polypeptide α-thioesters.* ***A****. Scheme representing the proposed mechanism for protein splicing involving intein VMA1.* ***B****. Expression, purification and cleavage of polypeptide-intein-CBD fusion protein (the asterisk refers to the mutation Asn454Ala in the intein element and CBD refers to the chitin bindinf domain) with an appropriate thiol.*

extein following an (S/O) →N acyl shift (similar to the last step of Native Chemical Ligation, see Figure 1A). Mutation of the conserved Asn residue within the intein to Ala blocks the splicing process in midstream thus resulting in the formation of an α-thioester linkage between *N*-extein and the intein [31] (Figure 2B). This thioester bond can be cleaved using an appropriate thiol through a trans-thioesterfication step to give the corresponding recombinant polypeptide α-thioester. The IMPACT expression system, commercially available from New England Biolabs [32,33], allows the generation of recombinant α-thioester proteins by making use of such modified inteins in conjunction with a chitin binding domain (CBD) for easy purification by affinity chromatography (see Figure 2B).

*Recombinant N-terminal Cys-containing polypeptides.* The introduction of *N*-terminal Cys residues into expressed proteins can be readily accomplished by cleaving (by proteolysis or auto-proteolysis) the appropriate fusion proteins. The simplest way to generate a recombinant polypeptide containing a *N*-terminal Cys residue is to introduce a Cys downstream to the initiating Met residue. Once the translation step is completed, the endogeneous methionyl aminopeptidases (MAP) removes the Met residue, thereby generating *in vivo* a *N*-terminal Cys residue [14,34-37]. Other approaches involve the use of exogenous proteases. Verdine and co-workers added a Factor Xa recognition sequence immediately in front of the *N*-terminal Cys residue of the protein of interest [38]. After purification, the fusion protein was treated with the protease Factor Xa which generated the corresponding *N*-terminal Cys protein. Tolbert and Wong have also showed that the cysteine

protease from tobacco etch virus (TEV) can also be used for the same purpose [39]. This protease is highly specific and it can be overexpressed in *E. coli*. Other proteases that cleave at the C-terminal side of their recognition site, like enterokinase and ubiquitin *C*-terminal hydrolase, could be also used for the generation of *N*-terminal Cys residues.

Protein splicing can also be engineered to produce recombinant *N*-terminal Cys-containing polypeptides. Several inteins have been already mutated in such a way that cleavage at the *C*-terminal splice junction (i.e. between the intein and the C-extein, see Figure 2B) can be accomplished in a pH- and temperature-dependent fashion [40-42].

**Biosynthesis of backbone cyclized polypeptides through intramolecular EPL**

The approach employed for the biosynthesis of backbone cyclized polypeptides using EPL is depicted in Figure 3. The target polypeptide to be cyclized is fused at the *N*-terminus with a peptide leading sequence immediately followed by a Cys residue, and at the *C*-terminus with an engineered intein. The *N*-terminal leading sequence can be cleaved *in vitro* or *in vivo* by a proteolytic or self-proteolytic event thereby generating the required *N*-terminal Cys residue. This Cys residue then reacts in an intramolecular fashion with the α-thioester generated by the engineered intein at the *C*-terminus thus providing a recombinantly generated backbone cyclized polypeptide. This approach has been used for the *in vitro* and *in vivo* biosynthesis of different backbone cyclized polypeptides.

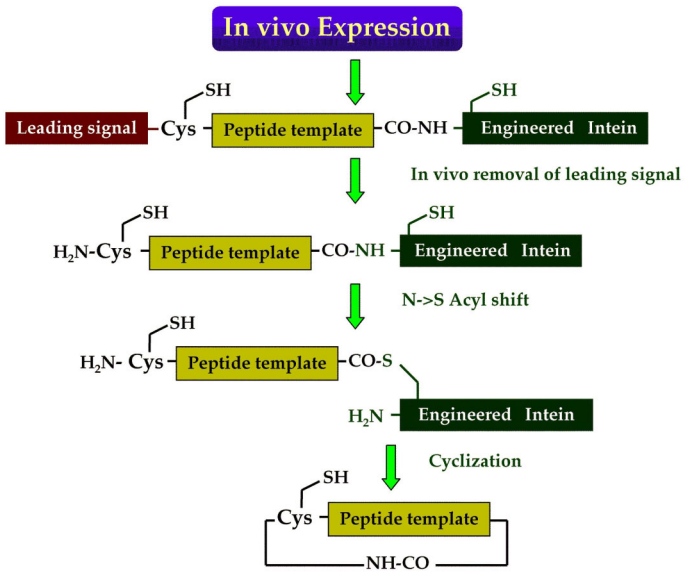


**Figure 3.** Scheme for the biosynthesis of cyclic polypeptides using intramolecular expressed protein ligation.

*In vitro biosynthesis*. The demonstration of this biosynthetic cyclization strategy was first reported *in vitro* by Camarero and Muir in 1999 using the *N*-terminal SH3 domain of the c-Crk protein as model protein [11]. In this work, the SH3 domain was fused to a modified VMA intein at the *C*-terminus and to the Met-Ile-Glu-Gly-Arg-Cys motif (which contains a Factor Xa proteolysis site) at the *N*-terminus. After expression in *E. coli* and purification, the intein fusion protein was treated with Factor Xa protease. This proteolytic step afforded a *N*-terminal Cys-containing SH3-intein fusion protein which spontaneously reacted in an intramolecular fashion to yield the corresponding cyclized SH3 domain. The cyclization process was extremely clean and fast, and the resulting cyclic SH3 protein domain was fully active [43]. Intriguingly, this intramolecular process did not require the presence of a thiol cofactor (absolutely necessary to facilitate intermolecular ligation reactions [24]).

This interesting result was explained on basis of the close proximity of both reacting groups in the folded state of the SH3 domain (the *N*- and *C*-termini of the natively folded SH3 are located within 6Å), which was able to increase the local concentration of both reacting groups. This effect has been already reported in the cyclization of different small protein domains [5,7].

Iwai and Pluckthum have also reported the biosysthesis of a cyclized version of the β-lactamase protein using a similar approach [36]. In their case, the *N*-terminal Cys residue was generated *in vivo* by removal of the initiating Met residue by an endogenous Met amino peptidase. After purification of the *N*-terminal Cys-containing intein fusion protein at pH 8.0, the cyclization was triggered by addition of a thiol cofactor at pH 5.0. The resulting cyclized protein was found to be more stable against irreversible denaturation upon heating than the linear form.

Evans and Xu have also described the concomitant use of two engineered inteins to obtain several biosynthetic backbone cyclized proteins [13]. In their approach, the intein at the *N*-terminus of the target polypeptide was designed to block splicing but allow C-terminal cleavage which provided the required *N*-terminal Cys residue. After purification of the corresponding fusion proteins, the *N*-terminal Cys residue was introduced by inducing the cleavage of the *N*-terminal modified intein by a change in temperature and pH of the buffer. The cyclization itself was achieved when a thiol cofactor was added to the *N*-terminal Cys fusion protein. In all the cases reported, the cyclic product was always obtained with significant amounts of uncyclized linear polypeptides.

It is interesting to remark that in the last two cases, the cyclization reaction did not occur spontaneously like in the SH3 protein domain and it required the presence of a thiol cofactor. A potential explanation for the different reactivity observed in the cyclization of these proteins could be found in the extremely long linkers (ranging from 12 to 26 residues) that were used to stabilize the new loops formed during the cyclization step. Although it is very well established that the use of such long linkers may help to stabilize the formation of a new loop by reducing strain, it also increases the average distance between the two reacting groups making the cyclization reaction kinetically less favorable [43]. In the case of the SH3 cyclization, only three residues (Gly-Cys-Gly) were added to release the stress introduced during the cyclization. This fact has been proved to be critical in order to allow the *in vivo* biosynthesis of cyclic polypeptides using intramolecular EPL.

*In vivo biosynthesis*. Based on the high efficiency observed during the *in vitro* cyclization of the SH3 domain [11,43], Camarero and Muir used a similar approach to test the possibility of carrying out the cyclization of the SH3 domain inside living cells. For this purpose, the Factor Xa recognition leading sequence in the SH3-VMA intein fusion protein was replaced by a Met residue. During the expression of the resulting fusion protein in *E. coli* cells, the Met residue was efficiently removed by an endogenous Met aminopeptidase [34], This *in vivo* proteolytic event unmasked the *N*-terminal Cys residue which then reacted in an intramolecular fashion with the α-thioester group induced by the C-terminal engineered VMA intein [14]. Analysis by SDS-PAGE showed that most of the SH3-intein fusion protein (>90%) was cleaved *in vivo*. Remarkably, when the entire soluble cell

fraction was analyzed by reverse-phase HPLC, the expected cyclic SH3 protein and the cleaved intein were found to be the major components in the mixture. It is worth noting that no linear SH3 domain was found in the cellular mixture, suggesting that *in vivo* hydrolysis of the α-thioester linkage present in the precursor protein was minimal. This work demonstrated the first example of a polypeptide chemical ligation reaction performed in the complex cytoplasmic environment of a living cell, and represents an important milestone in current efforts to generate and screen libraries of cyclic polypeptides inside living cells.

The only alternative approach to EPL for the biosynthesis of cyclic polypeptides *in vivo* is the use of protein trans-splicing. However, these systems require the presence of specific amino acid residues at both intein-extein junctions for efficient protein splicing to occur [12,16,44]. In contrast to protein trans-splicing, the only absolute sequence requirements for native chemical ligation is the presence of a *N*-terminal cysteine. Model studies have also shown that all 20 natural amino acids located at the *C*-terminus of a polypeptide α-thioester can support ligation [45]. Moreover, the engineered inteins used to generate recombinant polypeptide α-thioesters are compatible with most amino acids upstream of the cleavage site [17]. Thus, our native chemical ligation approach may be quite general with respect to the sequence of the linear peptide precursor and is potentially a powerful tool to generate a diverse array of backbone cyclized polypeptides *in vivo*.

**In vivo biosynthesis of cyclic polypeptide-based libraries**

The ability to create cyclic polypeptides *in vivo* opens up the possibility of generating large libraries of cyclic polypeptides. Using the tools of molecular biology, genetically encoded libraries of cyclic polypeptides containing billions of members can be readily generated. This tremendous molecular diversity forms the basis for selection strategies that model natural evolutionary processes. Also, since the cyclic polypeptides are generated inside living cells, these libraries can be directly screened for their ability to attenuate or inhibit cellular processes.

In contrast to phage display, where the screening takes place *in vitro*, screening that takes place in the cytoplasm offers the advantages conferred by a native physiological environment where diverse biochemical events may be examined. In addition, problems resulting from the presence of a fusion tag (in this case the viral particle), in a phenomenon known as template effect, may be circumvented.

Backbone cyclized polypeptides are relatively more stable and more resistant to cellular catabolism than linear polypeptides or disulfide-based cyclic polypeptides. Naturally occurring cyclic peptides often exhibit diverse therapeutic activities ranging from immunosuppression to antimicrobial activity. The stability of backbone cyclized polypeptides that display certain pharmacological properties suggests that they may be suitable scaffolds on which to graft the molecular diversity of an intracellular library [44,46].

A number of advances in *in vivo* library generation and screening have recently been made. Scott and Benkovic used protein trans-splicing to generate the cyclic peptide Pseudostellarin F, an eight amino acid circular peptide with tyrosinase inhibitory activity [12]. The *in vivo* biosynthesized Pseudostellarin F was fully active and

successfully screened *in vivo* for its tyrosinase inhibitory activity. More recently, cyclic peptide libraries based on the Pseudostellarin F scaffold demonstrated the structural requirements for this system. Apparently, several amino acids positions near the intein-extein junction are critical for expression and cyclization and the authors estimated that 70% of their library produced cyclic products.

Payan and coworkers also used a similar protein trans-splicing approach to generate random cyclic peptides in the cytoplasm of human cells using a retroviral expression vector [47]. Screening of the library for modulation of the IL-4 signaling pathway, led to the identification of several cyclic peptides that selectively inhibit the ε promoter activity. The library was based upon a five amino acid coding strategy, and the potential complexity of the library was about 160,000 members at the amino acids level. Of the 565 clones tested, twenty-three hits were identified. These small circular peptides are potential therapeutic agents against allergy and asthma and may serve in the future as leads for the development of more potent compounds. These results demonstrate an efficient functional screen for cyclic peptides *in vivo* in mammalian cells.

**Conclusions and Remarks.**

The ability to biosynthesize backbone cyclic peptides using EPL or protein trans-splicing has important implications for drug-development efforts. The capability to screen for biochemical events in an environment as complex as the cell's interior will result in valuable and unique information about potential leads identified by this method. Indeed, peptide-based libraries have been already shown to be effective in producing drug candidates in bacterial as well as mammalian systems [12,47].

In summary, we have reviewed recent developments on the use of engineered inteins for the biosythesis of circular polypeptides. Protein trans-splicing has revealed itself as a powerful tool for the biosynthesis of circular polypeptides that include small peptides and large proteins [12,15,19]. It also has been shown that this approach can be used for the generation of large libraries of circular polypeptide inside living cells where they can be directly screened for biological activity [16,47]. However, it has been shown that specific residues of the native *N*-extein and *C*-extein are required for efficient protein trans-splicing to occur with the naturally split DnaE intein [19]. It is conceivable, therefore, that this could restrict or bias the sequence diversity in the corresponding circular peptide libraries generated by this method.

Intramolecular EPL, on the other hand, has also been used successfully for the *in vitro* and *in vivo* biosynthesis of cyclic polypeptides [11,13,14,36,43]. In contrast with the protein trans-splicing approach, EPL is compatible with most of the amino acids at the cyclization site [17,45], making this approach general with respect of the sequence of the linear polypeptide sequence.

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